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Sensitive determination of isoprostanes in exhaled breath condensate samples with use of liquid chromatography–tandem mass spectrometry

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ABSTRACT

Oxidative stress is the hallmark of various inflammatory lung diseases. Increased concentrations of reactive oxygen species in the lungs are reflected by elevated concentrations of oxidative stress markers in the breath, airways, lung tissue and blood. The aim of this work was to develop a method for the fast measurement of F2-isoprostanes in exhaled breath condensate (EBC) samples using equipment which is nowadays available and routinely exploited in analytical laboratories, liquid chromatography coupled with tandem mass spectrometry. Because of the limited volume of an EBC sample and the very low concentrations of biomarkers, we chose lyophilization as the preconcentration technique. The diastereoisomers determined show similar fragmentation patterns, which is why complete chromatographic separation with excellent peak shapes was essential for accurate quantitation. Isoprostanes were separated using a narrow-bore Agilent Extend C-18 column in isocratic elution mode using acetonitrile/methanol and water with the addition of 0.01%(v/v) formic acid. The limits of determination and quantitation for the determination of four isoprostanes in samples of EBC ranged from 1 to 3 pg/ml. The recoveries of all isoprostanes ranged from 96.7 to 101.7, with a relative standard deviation of <7%. The stability of the isoprostanes at different temperatures was measured as well.

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1. Introduction

Oxidative stress (OS) is a state of imbalance between continuously generating reactive oxygen species (ROS) during normal cell metabolism and the mechanism for detoxifying oxygen radicals by a network of antioxidative enzymes [1]. Products of ROS activity are measured as biomarkers of oxidative stress. Abnormal levels of OS biomarkers are observed in various acute and chronic diseases, e.g., cancer, cardiovascular disease, neurodegenerative disease, lung disease and even the normal aging process [2–6].

Owing to its sensitivity, chemical stability and reliability as an index of lipid peroxidation, one of the most commonly studied markers is 8-iso-PGF$_{2\alpha}$ [7,8]. This substance and its isomers are measured in different biological specimen like urine, plasma, cerebrospinal fluid, liver, kidneys, brain tissue, bronchopulmonary lavage, induced sputum and exhaled breath condensates (EBC) [9–16].

Traditionally, the measurement of lung disease biomarkers has involved invasive procedures to procure samples or to examine the affected compartments, which causes the patient discomfort. The collection of exhaled breath condensate (EBC) has recently emerged as a non-invasive sampling method for the real-time analysis and evaluation of oxidative stress biomarkers in the lower respiratory tract airways. The main advantages of this method are that it is non-invasive, convenient, and can be carried out on mechanically ventilated patients as well as on children. The limitations of EBC sample analysis include the small volume of a sample, high levels of interfering substances, relatively low concentrations of biomarkers, and the lack of a standard collection method.

The quantification of isoprostanes is a reliable marker of lipid peroxidation in vivo [17–19], and several techniques for this are currently used [20–22,7,23–25], including: gas chromatography (GC)–mass spectrometry (MS) associated with immunoaffinity extraction, GC–tandem MS, and liquid chromatography–tandem MS.

In this study we investigated the potential usefulness of the HPLC–MS/MS procedure for the accurate qualitative analysis of EBC. A new procedure was developed for determining the content of four isoprostanes in samples of EBC. Sample preparation was based on the lyophilization of EBC samples and the dissolution of the resultant dry residue, prior to analysis with LC–MS/MS.

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Since the majority of laboratories are equipped with an HPLC–MS/MS system, this procedure can be routinely used for the determination of isoprostanes in EBC samples.

2. Materials and methods

2.1. Chemicals

HPLC–MS grade methanol and acetonitrile were obtained from Witko (Łódź, Poland), formic acid from POCh (Gliwice, Poland). Ultrapure water was produced in an HPLC5 system (Hydrolab, Poland).

Authentic standards of isoprostanes (8-iso-$PGF_{2\alpha}$, 8-iso-15(R) $PGF_{2\alpha}$, 11-$PGF_{2\alpha}$ and 15(R)-$PGF_{2\alpha}$) were obtained from SPI-BIO (Montigny le Bretonneux, France), as was the deuterated internal standard 8-iso-$PGF_{2\alpha}$-d_4.

2.2. Standard solutions

The stock solutions of 8-iso-$PGF_{2\alpha}$ (8-isoP), 8-iso-15(R)$PGF_{2\alpha}$ (8,15-isoP), 11-$PGF_{2\alpha}$ (11-isoP) and 15(R)-$PGF_{2\alpha}$ (15-isoP) – 1 ng/mL of each – were prepared by diluting the standards in methanol and then storing them at −80 °C.

A series of calibration solutions were prepared by diluting the intermediate solutions with methanol:acetonitrile:water (25:25:50 v/v/v) in the concentration range 1–200 pg/mL.

The chemical structures of the four isoprostanes and the internal standard are shown in Fig. 1.

2.3. Sample preparation

Exhaled breath condensate samples were collected from students of the Gdańsk University of Technology (GUT) using a TurboDeccs (Italy) device. The subjects, wearing a nose-clip, breathed tidally through a mouthpiece connected to a saliva trap. One to two milliliters of condensate were collected after 10 min. 1 ml of the EBC sample was spiked with the internal standard and stored at −80 °C. The frozen samples were lyophilized to dryness, reconstituted in 200 μl mobile phase (H_2O:ACN/MeOH 1:1) and analyzed (Fig. 2).

2.4. HPLC–MS/MS analysis

HPLC analyses were performed on an Agilent 1200 HPLC system. The chromatographic system consisted of a G1367 C autosampler (injection volume 100 μl), a G1312B binary pump and a G1316B thermostatted column. The analytes were separated on an Agilent Extend C-18 column (50 mm × 2.1 mm; 1.8 μm). The mobile phases A (methanol/acetonitrile 1:1) and B (ultrapure water) contained 0.01% (v/v) formic acid at a flow rate of 0.35 ml/min. The column was maintained at 40 °C.

The HPLC system was coupled directly to a triple quadrupole mass spectrometer (API 4000; Applied Biosystems, Darmstadt, Germany). The mass spectrometer was optimized in MRM mode by diffusing 50 ng/ml of each individual isoprostane standard solution. The instrument was operated in negative ion mode with the declustering potential at 115 eV, focusing potential at 200 eV, entrance potential at 10 eV, collision energy at 33 eV, collision cell exit potential at 15 eV, ion spray voltage at 4500 V and source temperature at 550 °C. The most abundant product ion of all the analytes was found at *m/z* 193, the mass transition of $[M{-}H^+]^-$ *m/z* 353 in Q1 to *m/z* 193 in Q3 was selected for the quantitative analysis during LC–MS/MS, and the corresponding transition of *m/z* 357–197 was monitored for the deuterated internal standard.

Table 1
Calibration data of four isoprostanes obtained using an Agilent Extend C-18 column (50 mm × 2.1 mm; 1.8 μm).

Analyte	Curve equation	R^2	LOD	LOQ
8,15-isoP	$y = 0.0043x + 0.109$	0.999	2	7
8-isoP	$y = 0.00493x + 0.0836$	0.999	1	4
11-isoP	$y = 0.00337x + 0.0364$	0.999	5	17
15-isoP	$y = 0.00415x + 0.000862$	0.999	3	10

Instrument control, data acquisition and data analysis were carried out with Analyst Software 1.5.2 (Applied Biosystems).

3. Results and discussion

Fig. 3 shows the mass spectrum of 8,15-isoP, because 8-isoP, 8,15-isoP, 11-isoP and 15-isoP are diastereoisomers and they all have similar fragmentation patterns. Because of this, complete chromatographic separation with excellent peak shapes is essential for accurate quantitation. Method selectivity was achieved by a combination of retention time, precursor ion and product ions.

The proposed HPLC procedures enabled the quantitation of 8-isoP, 8,15-isoP, 11-isoP and 15-isoP in EBC samples with tandem MS as the detection system. Applying the chromatographic conditions mentioned above to a narrow-bore column, the retention times for IS and the four isoprostanes were 4.77, 4.47, 4.77, 5.15 and 6.01 min, respectively. Fig. 4 shows a chromatogram of an EBC sample spiked with isoprostanes.

3.1. Method validation

The stock solutions were used for the preparation of eight calibration solutions at concentrations of 5, 10, 25, 50, 75, 100, 150, and 200 pg/ml. The calibration solutions were spiked with the IS (8-iso-$PGF_{2\alpha}$-d_4). The calibration curves were plotted using the dependence of the ratio of the peak area of the particular substance (8-isoP, 8,15-isoP, 11-isoP, 15-isoP) to the peak area of the IS (8-iso-$PGF_{2\alpha}$-d_4) on the concentration of a particular biomarker. The analysis of each calibration solution was repeated three times. Limits of detection (LODs) and limits of quantitation (LOQs) were determined by serial dilution of standard solutions. LODs and LOQs were evaluated on the basis of respective signal-to-noise ratios of 3 and 10. The calibration data, including calibration line equations, coefficients of determination, limits of detection and quantitation, are listed in Table 1.

Calibration curves were linear within the studied range of concentrations with coefficients of determination of >0.998 for all isoprostanes.

The trueness, repeatability and reproducibility of the method were tested with fortified samples of exhaled breath condensates.

To determine trueness, samples of EBC were spiked at different concentration levels (25, 100, 250 pg/mL). Every sample was analyzed in triplicate according to the above procedure. The results were obtained by comparison of the peak areas of the spiked and

Table 2
Recoveries (%) and relative standard deviations RSD (%) obtained by LC–MS/MS analysis of EBC samples fortified with standard solutions of four isoprostanes at three spiking levels (25, 100 and 250 pg/mL).

Compound	Recovery (%) of three concentration (RSD) ($n = 3$)		
	25 pg/mL	100 pg/mL	250 pg/mL
8,15-isoP	97.0(4.7)	101.7(3.5)	98.8(2.1)
8-isoP	98.5(2.5)	98.9(3.6)	97.7(1.9)
11-isoP	100.6(6.6)	98.9(2.2)	98.4(2.5)
15-isoP	96.7(6.3)	99.6(2.8)	97.9(1.9)

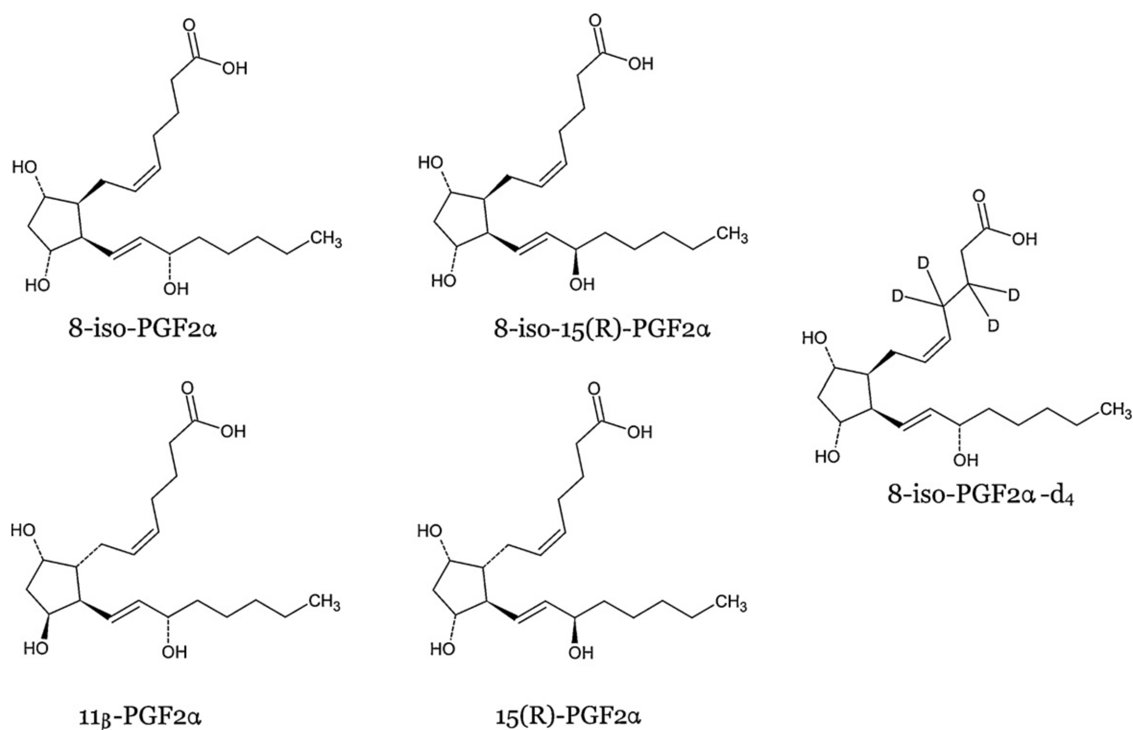

Fig. 1. Chemical structures of determined isoprostanes and of the internal standard (8-iso-$PGF_{2\alpha}$-d_4).

processed EBC samples with the corresponding standard solutions (matrix free) analyzed without lyophilization (see Table 2).

Satisfactory recoveries (96.7–101.7%), with relative standard deviations (RSDs) <7%, were obtained for all isoprostanes, regardless of the spiking level.

The repeatability and reproducibility of the method were assessed by replicate analyses of EBC samples at a chosen spiking level (200 pg/mL). The samples of EBC were analyzed three times a day (nine samples of EBC) to determine the intra-day repeatability expressed as RSD within-day averages. The analyses were repeated for three consecutive days to calculate inter-day reproducibility expressed as RSD between-day averages. The results of these studies are given in Table 3.

The intra-day repeatability was between 6.4 and 9.4%, and the inter-day reproducibility was between 4.5 and 5.8%.

3.2. *Sample stability*

The stability of oxidative stress markers is closely related to the matrix composition of the sample. Since most samples are usually

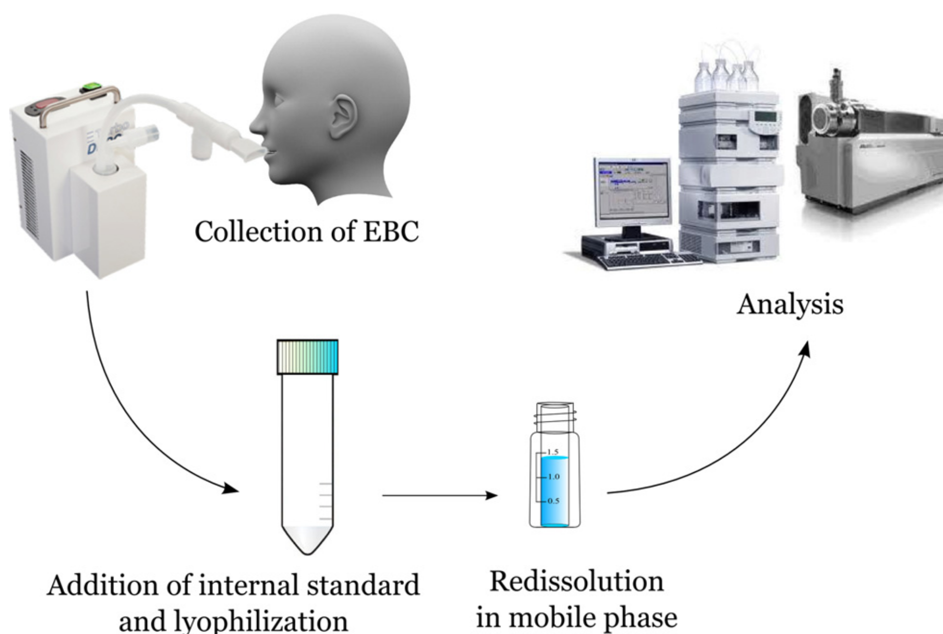

Fig. 2. Scheme showing the collection, preparation and analysis of EBC samples.

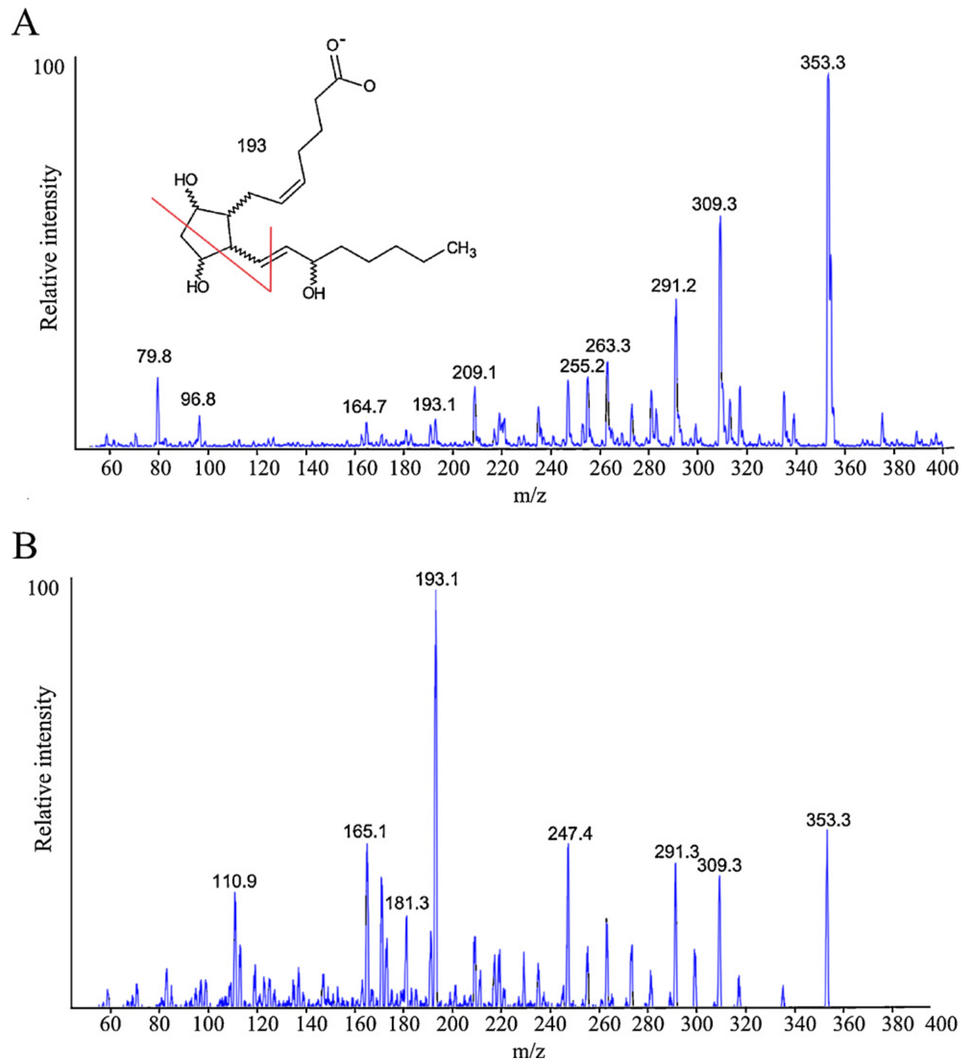

Fig. 3. Mass spectrum of 8,15-isoP: (A) full scan with proposed fragmentation pattern, (B) spectrum of product ion *m/z* 353.3 with selected quantitation fragment ion *m/z* 193.3.

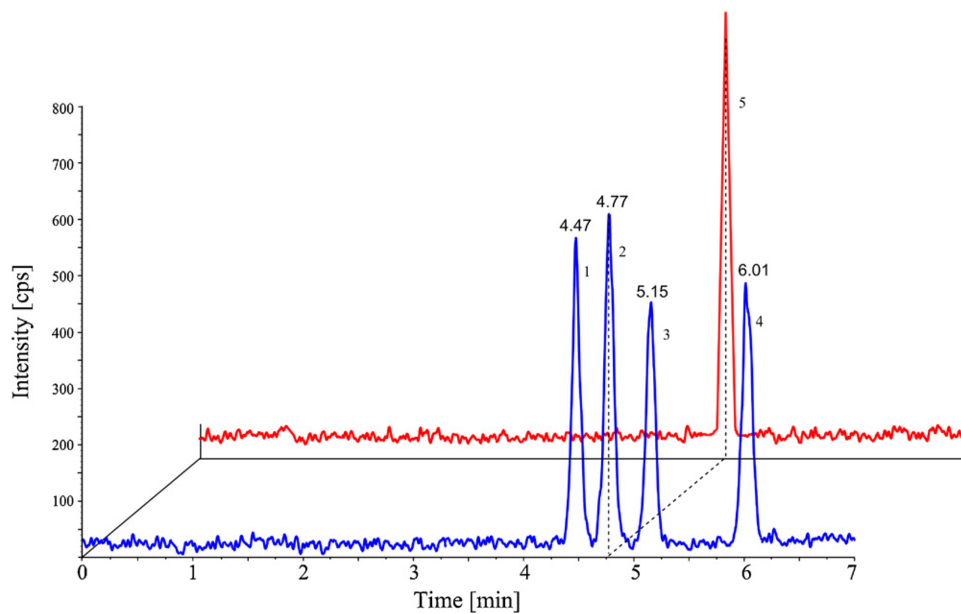

Fig. 4. LC–MS/MS chromatogram of standards (100 pg/ml) and internal standard showing chromatographic separation ((1) 8-iso-15(R)PGF$_{2\alpha}$; (2) 8-iso-PGF$_{2\alpha}$; (3) 11-PGF$_{2\alpha}$; (4) 15(R)-PGF$_{2\alpha}$, (5) 8-iso-PGF$_{2\alpha}$-d$_4$).

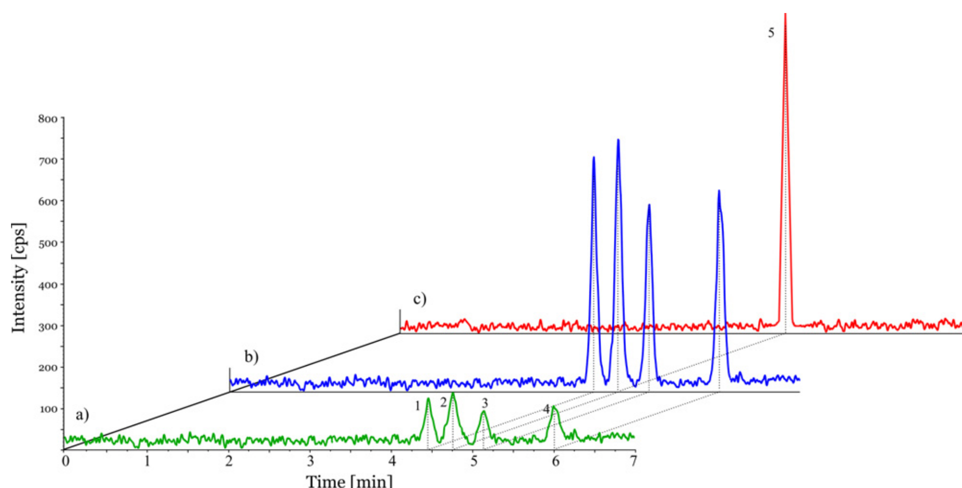

Fig. 5. LC–MS/MS chromatogram of (a) a subject's EBC sample (concentration of isoprostanes ∼ 15 pg/mL) showing chromatographic separation ((1) 8-iso-15(R)-PGF$_{2\alpha}$; (2) 8-iso-PGF$_{2\alpha}$; (3) 11-PGF$_{2\alpha}$; (4) 15(R)-PGF$_{2\alpha}$), (b) standards of four isoprostanes (100 pg/ml), (c) internal standard 8-iso-PGF$_{2\alpha}$-d$_4$.

stored before final analysis, the stability of biomarkers of interest should be examined under different conditions. Samples such as blood plasma or solid tissues frozen in liquid nitrogen and stored at −80 °C did not undergo auto-oxidation for up to eight months [26]. But changing the conditions of blood plasma storage to +4 °C resulted in increased levels of isoprostanes [27]. This must have been due to the auto-oxidation of plasma lipids resulting from the artificial generation of isoprostanes.

The matrix of EBC samples is a relatively simple one, so auto-oxidation processes should generate fewer auto-oxidation products than samples containing large amounts of lipids, proteins and salts.

3.2.1. Stability of EBC samples after one, two and three freeze–thaw cycles

The variation of isoprostane concentrations when QC samples were subjected to three successive freeze–thaw cycles was checked: the results indicate that there is no significant loss of isoprostanes after up to three freeze–thaw cycles.

3.2.2. Stability of aqueous samples in HPLC vials (i.e. ready for HPLC analysis) at −80 °C, −20 °C, +4 °C and +20 °C

99.9% of an EBC sample consists of water. Stability studies of aqueous samples spiked with isoprostanes were carried out. Samples were left at four different temperatures and analyzed during a period of 8 weeks. To compensate changes in detector's response, a fresh portion of deuterated internal standard was added to each sample before analysis. The variations in isoprostane levels at concentrations of 250 pg/ml of each were <11.1%.

Table 3
Intra-day repeatability and inter-day reproducibility of the assay, performed three times a day on three consecutive days.

Analyte	Recovery (%) (RSD) (n = 3)			Inter-day
	Intra-day (n = 3)			
	Day 1	Day 2	Day 3	
8,15-isoP	98.3(9.4)	101.7(8.3)	92.0(7.8)	97.3(4.9)
8-isoP	101.3(9.0)	104.8(8.0)	95.9(9.0)	100.6(4.5)
11-isoP	100.6(7.2)	104.1(7.5)	93.5(6.4)	99.4(5.4)
15-isoP	100.9(7.2)	103.8(6.7)	92.7(7.5)	99.1(5.8)

3.2.3. Stability of EBC samples after 6 h of storage at +20 °C

In contrast to the stability studies done by Syslová et al. [28], we measured the concentration of four isoprostanes in EBC samples by LC–MS/MS; held at room temperature until processing, the samples remained essentially unchanged.

3.2.4. Stability of EBC samples after two weeks of storage at −80 °C,+4 °C and +20 °C

Stability studies done during two weeks showed no significant loss of isoprostanes. At −80 °C, the substances were stable for the experimentally evaluated period. The matrix effect on the stability of isoprostanes in water and EBC below −80 °C was negligible.

This study shows that multiple EBC samples can be analyzed within a safe period of time without significant loss of isoprostanes.

3.3. Analysis of exhaled breath condensate samples

In this study a narrow-bore column of 2.1 mm internal diameter and 1.8 μm particle size was used. Sharper, more intense peaks were obtained with a small column ID and particle size than with standard-bore columns, the latter giving lower limits of detection and quantitation. Narrower column width results in lower LC flow rate. This approach allows to use less amounts of reagents and consequently produces less waste to dispose. Such a column also allows an isocratic elution program to be used, which gives excellent resolution of peaks with shorter retention times. There is, moreover, no need to equilibrate the column, and a larger number of samples can be analyzed in a shorter time.

The main drawback of narrow-bore columns in comparison with standard-bore columns is their reduced capacity: since fewer stationary phase particles can physically fit into the narrower column, there is less surface area available for species to bind. As a result, these columns can easily become overloaded. Typically, such a reduced capacity is compensated by diluting the samples more or by using smaller injection volumes. This problem does not appear in the analysis of EBC samples, because the biomarker concentrations are at pg/mL levels.

To demonstrate the application of the method to biological sample analysis, a series of EBC samples collected from volunteers at the Gdańsk University of Technology were analyzed.

The results showed in Table 4 revealed elevated concentrations of isoprostanes in students suffering from asthma and in smokers (Fig. 5). Isoprostane concentrations in samples from healthy volunteers were below the LOD.

Table 4
Concentrations of isoprostanes in EBC samples of smokers and non-smokers.

Analyte	Concentration [pg/mL]	
	Non-smokers ($n = 43$)	Smokers ($n = 7$)
8-iso-15(R)-PGF2α	<LOD	8.5–179
8-iso-PGF2α		17–35
11β-PGF2α		13–27
15(R)-PGF2α		14–21

4. Conclusions

The collection of EBC is suggested as a simple, quick, safe, convenient and non-invasive means of obtaining samples from the lower respiratory tract of humans of any age with inflammatory lung disease. A wide range of constituents in EBC has been previously measured. Therefore, the analysis of EBC components may provide a means of monitoring airway inflammation and cellular metabolism of the lung. However, a number of important methodological issues need to be addressed, regardless of which EBC compound is being analyzed. These include repeatability, dilution, environmental contamination, effect of breathing patterns, and assay sensitivity. Another important problem is that the internal coating of the collection device may influence the levels of some constituents. It has been suggested that dilution markers may be important for some compounds. Direct comparison between different protocols is therefore complicated because of the absence of an appropriate standardization of sample collection and the varied specificity of the measurement methods.

In most published studies, F2-isoprostanes were measured using commercially available enzyme immunoassays [29–33]. However, the available ELISA kits were originally validated for matrices different from EBC, such as urine, plasma or serum. Quantification using ELISA kits may be influenced by a matrix containing dissolved analytes. Because of the extremely high dilution of the epithelial lining fluid in EBC (resulting in low biomarkers concentrations), EBC is thought to contain a much smaller amount of matrix than other highly concentrated and protein-rich matrices like plasma or urine. The results of a comprehensive comparative study, performed using two unrelated analytical techniques for the quantitation of the three most commonly analyzed eicosanoids, showed that analyte levels in EBC had been overestimated.

This study of the stability of isoprostanes in exhaled breath condensates demonstrated that they can be stored even at room temperature, for a short period of time, without significant impact on their recovery. However, EBC samples from each individual may vary in matrix composition, so to eliminate undesirable reactions involving isoprostanes, it is recommended to store samples at a temperature of $-20\,^{\circ}C$ or $-80\,^{\circ}C$.

A method for the detection and quantitation of 8-iso-$PGF_{2\alpha}$, 8-iso-15(R) $PGF_{2\alpha}$, 11-$PGF_{2\alpha}$ and 15(R)-$PGF_{2\alpha}$ as oxidative stress markers in exhaled breath condensate samples by LC–ESI-MS/MS was developed.

Proposed procedure is specific due to its ability to work in multiple reaction monitoring mode (MRM), where a specific set of precursor ions is selected and their transition from a selected precursor ion to a specific product ion is monitored. The MRM mode reduces chemical noise, leading to higher mass spectral sensitivity and selectivity, which is used for the quantitative determination. A deuterated internal standard is used to obtain a high quantitation precision by compensation of detector's response. The use of lyophilization for sample preparation resulted in target analyte enrichment and a high sample throughput. In order to circumvent the various problems in conventional assay methods, such as the laborious and time-consuming pretreatments in GC–MS and possible cross-reactions of the target compounds in enzyme immunoassays (EIA), liquid chromatography coupled with tandem mass spectrometry was used. Unlike GC–MS, this hyphenated technique does not require a two-step derivatization procedure: recovery is therefore improved, the sample pre-treatment time is shorter and the incomplete derivatization of by-products is avoided.

In the majority of published research only one isoprostane (8-isoP) was determined in EBC, plasma and urine samples [23,25]. The advantage of the method described in the present article is that four isoprostanes were determined. The different patterns of isoprostane concentrations in EBC samples could reveal new dependency between diseases.

The excellent accuracy, reproducibility, and high throughput of this method should enable it to be used in large clinical studies and standard clinical laboratories.

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